Identification of Mycoplasma felis and Mycoplasma gateae by an Immunobinding Assay†

MARY B. BROWN,†* PATRICIA GIONET,‡ AND DAVID F. SENIOR³

Departments of Infectious Diseases¹ and Small Animal Clinical Sciences,² College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610

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An immunobinding assay capable of distinguishing between Mycoplasma felis and Mycoplasma gateae was developed. Nitrocellulose was used as the solid support. Polyclonal rabbit antiserum against M. felis or M. gateae was used in the assay. Binding of the specific rabbit antiserum was detected by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and an alkaline phosphatase substrate (Nitro Blue Tetracyclolium–5-bromo-4-chloro-3-indolyl phosphate). The immunobinding assay was effective in the detection of feline mycoplasmas on agar plates, on primary isolation plates, in broth cultures, and in mixed cultures. No cross-reactions were observed with other related mycoplasmal species. The assay was cheap to perform and easy to interpret, and it required little technical time.

Both Mycoplasma felis and Mycoplasma gateae have been isolated from both clinically ill and clinically healthy domestic cats (3, 4, 18, 20, 21). Both mycoplasmal species were isolated most frequently from throat swabs but have also been recovered from urogenital, nasal, and conjunctival swabs (4, 18, 20, 21). An especially high isolation rate was found for colony-maintained cats (4). Although the pathogenic potential of mycoplasmas in the cat is poorly defined, mycoplasmas have been implicated in a variety of feline diseases, including respiratory disease, conjunctivitis, and arthritis (9, 12, 19, 21). Both M. felis and M. gateae can establish chronic, persistent infections in experimentally inoculated kittens (19). M. felis was associated with conjunctivitis in 6- to 10-week-old kittens, with subsequent respiratory involvement (19). Both M. felis and M. gateae have been isolated from joints of immunocompromised cats with polyarthritis (12, 14). The arthritis has been reproduced experimentally with M. gateae (14). In addition, M. felis has been implicated in pneumonitis in horses (15). A recent clinical report linked the presence of an unidentified Mycoplasma sp. with a lung abscess in a cat (9).

Unlike conventional bacteria, mycoplasmas rarely can be differentiated on the basis of biochemical tests. Presumptive species identification can be made on the basis of glucose fermentation, arginine or urea hydrolysis, hemadsorption, and phosphatase production (1, 2, 5, 8, 11, 18). Definitive species identification is performed by serological methods. The most commonly used techniques include metabolic inhibition, growth inhibition, or immunofluorescence (7, 10, 17). An immunobinding assay has been used for the detection of surface antigens and species identification of mycoplasmas which contaminate tissue cultures (6, 13). The objective of the present study was to develop an immunobinding assay for feline mycoplasmas and to apply the assay for detection of mycoplasmas in broth culture, on agar plates, and in mixed culture.

**Mycoplasmal reference strains and clinical isolates.** M. felis ATCC 23391, M. gateae ATCC 23392, M. feliminutum ATCC 25749, M. gallisepticum ATCC 19610, Acholeplasma laidlawii ATCC 23206, M. canis ATCC 19525, and M. arginini ATCC 23838 were used as reference strains. Additional reference strains of M. felis and M. gateae were a gift from the collection of J. G. Tully, Mycoplasma Section, Frederick Cancer Research Facility, National Institute of Allergy and Infectious Diseases. Clinical isolates of M. felis (n = 15) and M. gateae (n = 15) were obtained from throat swabs of cats presented to the University of Florida Veterinary Medical Teaching Hospital Clinic. Informed consent was obtained from owners before inclusion of a cat in the study. The clinical isolates were identified on the basis of glucose fermentation (16), arginine hydrolysis (2), metabolic inhibition (17, 18, 22), and growth inhibition (7). Unless noted otherwise, all strains were grown in SP4 broth or SP4 agar (23). Broth cultures were incubated at 37°C in ambient air; agar plates were incubated at 37°C in 5% CO2. Broth cultures containing mycoplasmas were stored at −70°C before use; agar plates containing mycoplasmas were stored at 4°C before use.

**Rabbit antiserum.** Polyclonal antiserum to the ATCC reference strains of M. felis and M. gateae were prepared as previously described (6). M. felis or M. gateae was grown in 1 liter of SP4 broth containing rabbit serum in lieu of fetal calf serum. For M. gateae, SP4 was supplemented with 1% arginine. Microorganisms were harvested in the mid-logarithmic stage of growth by centrifugation for 30 min at 10,000 × g in a Beckman JA-14 rotor. The cells were washed three times in sterile phosphate-buffered saline solution (PBS), suspended in 10 ml of PBS, and sonicated for 30 s at 50% power in a sonic dismembrator to disperse aggregates. The CFU were determined, and the suspension was divided among 10 1-ml vials and stored at −70°C. Two New Zealand White rabbits were immunized for each mycoplasmal species. Preimmunization serum was obtained as a control. Rabbits received four consecutive daily injections into the marginal ear vein, with increasing doses (1, 2, 3, and 4 ml) of M. felis or M. gateae at 103 CFU/ml. Rabbits were tested on day 17 for reactivity. Preimmunization serum served as a negative control. Antiserum was obtained at weekly intervals until the titers declined. The concentrations of antiserum and conjugate used in the assay were chosen on the basis of checkerboard titrations.

**Cross-reaction.** Rabbit antisera were tested for reactivity
M. felis
M. gateae

FIG. 1. Immunoblots of colonies of *M. felis* or *M. gateae* reacted with rabbit anti-*M. felis* antiserum (lane 1), rabbit anti-*M. gateae* antiserum (lane 2), or normal rabbit serum (lane 3).

with the homologous mycoplasma used as an immunogen as well as for cross-reactivity with heterologous mycoplasmas listed above. Mycoplasma species which were tested for cross-reactions were those species which (i) are commonly isolated from feline or canine sources or (ii) are either arginine hydrolyzers or glucose fermenters which have demonstrated cross-reactions in other systems (8, 17, 18).

**Immunobssay.** Unless noted otherwise, all reagents were purchased from Sigma Chemical Co., St. Louis, Mo. All incubation and washing steps were performed with continuous, gentle rotation on an orbital shaker at room temperature. The immunoblot assay was used for mycoplasmas grown in broth or on agar. For mixed cultures, *M. felis* and *M. gateae* with known CFU were mixed to give a ratio of approximately 1:10. The mixed culture was diluted and plated on SP4 to give individual colonies.

For the detection of colonies on agar, nitrocellulose (NC) was placed on the surface of the agar and pressed gently into contact with the colonies. The NC was removed with forceps and was placed in 0.1 M Tris, pH 7.6, containing 9% NaCl for 5 min. The NC was placed in 0.1 M Tris, pH 7.6, containing 9% NaCl, 10% horse serum, and 0.2% Tween 20 for 30 min to block unreacted protein-binding sites. The NC was washed three times in PBS (5 min per wash) and was transferred to rabbit anti-*M. felis* antiserum, anti-*M. gateae*, or normal rabbit serum diluted 1:500 in PBS. After a 30-min incubation, the NC was washed three times in Tris-buffered saline, and the NC was incubated for 30 min in alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Southern Biotechnology Association, Birmingham, Ala.) diluted 1:500 in PBS. The NC was washed three times in PBS. Fresh substrate was prepared for each assay. Ten microliters of BCIP (0.2 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml in 100 μl of dimethyl sulfoxide) was added to 10 ml of a Nitro Blue Tetrazolium solution (2 mg/ml in 100 mM Tris hydrochloride, pH 9.7) to make Nitro Blue Tetrazolium-BCIP alkaline phosphatase substrate. The NC was incubated in the substrate until color development was observed, usually within 3 min or less. The reaction was stopped by a wash in deionized water. The blot procedure for broth cultures was identical to that described above, with one exception. Ten to twenty microliters of broth was placed on the NC and allowed to air dry, and the procedure described above was used for all subsequent steps.

**Results.** The entire assay could be performed in less than 5 h. The small dark dots corresponding to detection of individual colonies of *M. felis* and *M. gateae* are shown in Fig. 1. The same technique was applied directly to primary isolation plates, with similar results. The species of clinical isolates were identified from broth culture by using the

FIG. 2. Immunoblots of broth cultures of clinical isolates of *M. felis* (lanes 1, 3, and 5) or *M. gateae* (lanes 2, 4, and 6) reacted with rabbit anti-*M. gateae* antiserum (lanes 1 and 2), rabbit anti-*M. felis* antiserum (lanes 3 and 4), or normal rabbit serum (lanes 5 and 6).
immunoblot technique. The clinical isolates had been identified presumptively as *M. felis* or *M. gateae* on the basis of glucose fermentation (*M. felis*) or arginine hydrolysis (*M. gateae*). Identification of the isolates was confirmed by growth inhibition and metabolic inhibition assays. A representative immunoblot strip demonstrating detection of clinical isolates of *M. felis* and *M. gateae* from broth cultures is shown in Fig. 2. With very few exceptions, immunoblot reactions of broth from clinical isolates were specific for one species only. In two cases, the broth cultures reacted with both anti-*M. felis* and anti-*M. gateae* antisera. In both cases, these were determined to be mixed cultures which could be separated into *M. felis* and *M. gateae* by streak plating followed by filter-cloning techniques. This implied that a primary isolation broth as well as a primary isolation plate can be used for species identification even when a mixed culture is present.

The ability to detect mixed colonies on an agar plate is demonstrated in Fig. 3. The individual colonies were distinct, and the relative ratio of species was estimated by the number of reactive colonies. As many as six different NC blots could be obtained from the location on the agar plate without appreciable loss of sensitivity. In addition, even after storage at 4°C for several weeks, the colonies were easily detected by the immunobinding procedure.

Broth cultures of heterologous mycoplasmas did not demonstrate cross-reactivity with either antiserum to *M. felis* or antiserum to *M. gateae* (Fig. 4). Similar results were observed with mycoplasmas grown on agar (data not shown). Thus, the assay was specific and did not cross-react with any heterologous *Mycoplasma* species known to be isolated from cats (18). Previous studies with feline and canine mycoplasmas have failed to demonstrate any cross-reactivity by growth inhibition, immunofluorescence, and metabolic inhibition (8, 17, 18). In one study (17), common antigens were detected by double immunodiffusion among arginine-hydrolyzing species and among glucose-fermenting species. These cross-reactions did not appear when strains were tested by the other methods. Because the immunoblot assay uses either intact cells as colonies or predominantly intact cells from broth culture, common cytoplasmic enzymes, such as those involved in arginine or glucose metabolism, might be undetected.

**Advantages of the immunoblot assay.** The immunobinding assay was effective in the detection of feline mycoplasmas on agar plates, on primary isolation plates, in broth cultures, and in mixed cultures. Although a 1:500 dilution was used in the assay, the antiserum could be diluted to >1:1,000 without loss of activity. This represents a considerable advantage over immunofluorescence, growth inhibition, and metabolic inhibition tests, which require much greater amounts of antiserum. An additional advantage over existing serological identification methods is that multiple strips of NC can be developed in a single assay.

The immunobinding assay represents significant advantages over immunofluorescence, the most sensitive and commonly used of the serological identification assays. The immunobinding assay is less labor intensive and can be completed within a few hours. Multiple samples can be run in the same time that a single sample can. No special equipment, such as a fluorescence microscope, is required. Because no radioactive components are used, the immunobinding assay can be adapted to any laboratory setting. Fresh colonies are not required for the assay. Since NC can be autoclaved, it is possible to identify specific colonies of interest for future subculture by using this method without contaminating the isolation plate. The immunobinding assay is adaptable to broth cultures, primary isolation plates, or agar subculture plates. The assay is cheap to perform, is easy to interpret, and requires little technical time relative to immunofluorescence.

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LITERATURE CITED